

## APPENDIX SHOWING CHANGES TO SPECIFICATION

Page 25, line 4:

### 9. N-box-[ ]GABP binding regulation

Page 25, line 8:

Mouse 3T3 cells were treated for 2 h with diethyl maleate (DEM), a glutathione (GSH)-depleting agent, in the presence or absence of N-acetylcysteine (NAC), an antioxidant and a precursor of GSH synthesis. Following treatment, the cells were harvested, and nuclear extracts were prepared in the absence of a reducing agent. GABP DNA binding activity was measured by EMSA analysis using oligonucleotide probes containing a single N-box (AGGAAG) or two tandem N-boxes (AGGAAGAGGAAG). Treatment of 3T3 cells with DEM resulted in a dramatic decrease in the formation of the GABP heterodimer ( $\text{GABP}\alpha\text{GABP}\beta$ , (Martin 1996<sup>89</sup>, Fig. 2A, lane 2) and heterotetramer ( $\text{GABP}\alpha_2\text{GABP}\beta_2$ ) (Ibid, Fig. 2A, lane 6) complexes on the single and double N-box. [X]Inhibition of GABP DNA binding activity by DEM treatment was prevented by simultaneous addition of NAC (Ibid, Fig. 2A, lane 4 and 8). The reduction of GABP DNA binding activity was not due to loss of GABP protein since the amount of  $\text{GABP}\alpha$  and  $\text{GABP}\beta_1$  was unaffected by DEM or NAC treatment. Dithiothreitol (DTT) is an antioxidant. DTT treatment of nuclear extracts prepared from DEM-treated 3T3 cells restored GABP binding activity. Treatment of 3T3 nuclear extracts with 5 mM GSSG nearly abolished GABP DNA binding. Based on these observations Martin *et al.*, concluded that GABP DNA binding activity is inhibited by oxidative stress, i.e. GSH depletion. The study also measured the effect of DEM treatment on expression of transiently transfected luciferase reporter constructs containing a TATA box with either upstream double N-box or C/EBP binding site (Ibid, Fig. 4). DEM treatment had no effect on luciferase expression from C/EBP-TA-Luc after 6 or 8 h treatment (Ibid, Fig. 4). However, DEM treatment of cells transfected with double N-box-TATA-Luc, resulted in a 28% decrease in luciferase expression after 6 h and a 62% decrease

after 8 h (Ibid, Fig. 4). Based on these results, Martin *et al.*, concluded that glutathione depletion inhibits GABP DNA binding activity resulting in reduced expression of GABP-regulated genes.

Page 26, line 6:

Microcompetition for GABP also decreases binding of GABP to the N-box. [X]Take a GABP gene sensitive to oxidative stress through GABP only<sup>1</sup>. The effect of microcompetition on the transcription of this gene is similar to the effect of oxidative stress. In other words, for this gene, microcompetition can be viewed as “excess oxidative stress.”

Page 31, line 27:

The Rb promoter includes a N-box at (-198,-193). Several plasmids were produced. pXRP1 included the normal (-686,-4) segment of the Rb promoter. pXRP3 included the same segment with a mutated N-box. [X]RBF-1x4 included 4 copies of the Rb N-box as promoter. All promoters controled the expression of the luciferase (luc) reporter gene. Cotransfection of hGABP $\alpha$  and hGABP $\beta_1$  expression plasmids with pXRP1 into SL2 Drosophila cells showed a 10-fold increase in the reporter gene activity. Cotransfection with RBF-1x4 showed a 13-fold increase. Cotransfection with pXRP3, the mutated N-box, showed no increase (Sowa 1997<sup>95</sup>). Based on these observations, and other results, Sowa, *et al.*, concluded that hGABP has a strong transactivating effect on the Rb gene promoter, suggesting that hGABP is the main transcativator for the core promoter element of the Rb gene.

Page 33, line 23:

A study used DNase I footprinting to map the sites of protein-DNA interaction on the (-383 to +8) fragment of the TF promoter. The study used nuclear extracts prepared from uninduced and lipopolysaccharide-induced THP-1 monocytic cells. Six regions were identified. Region number 7 (-363 to -343) and region number 2 (-191 to -172) contain an N-box. [X]THP-1 extracts formed two complexes on a consensus N-box. [X]Both complexes were competed with excess unlabeled N-box and 200-fold excess of a (-363 to -343) probe. The (-191 to -172)

probe, although not as effective as the (-363 to -343) probe, showed approximately 30% reduction in the N-box complex formation (Donovan-Peluso 1994<sup>98</sup>, Fig. 9).

Page 35, line 1:

The following study shows that the (-363 to -343) factor(s) repress TF transcription. Holzmüller, *et al.*, (1999<sup>101</sup>) call the (-363 to -343) fragment of the TF promoter the Py-box. [X]A deletion of the 5'-half of the Py-box increased expression of a luciferase reporter gene (Ibid, Fig. 3A and B). The relative increase was similar for LPS induced or nontreated cells and was independent of the existence of NF- $\kappa$ B site (Ibid, Fig. 3C). Mutation of the N-box part of the Py-box resulted in complete loss of binding activity to the Py-box.

Page 44, line 1:

Consider information about the N-box. [X]The region -780 bp 5' of exon B to the start of exon 1 was suggested to include potential regulatory sites of the human HSL gene in adipocytes (Talmud 1998<sup>123</sup>, Grober 1997<sup>124</sup>). This region includes 15 N-boxes. Moreover, three pairs are located at short distances of each other. The distance between the pair at (+268,+272), (+279,+285) is 5 bp or 1.0 helical turn (HT), at (+936,+942), (+964,+970) is 22 bp or 2.5 HT, and at (+1,253,+1259), (+1270,+1276) is 11 bp or 1.5 HT.

Page 67, line 1- 6:

Oxidative stress reduces the binding of GABP $\alpha$  to the N-box. [X]Assume the propulsion genes, TF, CD18 and  $\alpha_4$  integrin, are responsive to oxidative stress exclusively through GABP. GABP stimulates CD18 and  $\alpha_4$  integrin transcription. Reduced binding of GABP $\alpha$  to DNA decreased CD18 and  $\alpha_4$  integrin transcription resulting is diminished forward motility. On the other hand, GABP represses TF transcription, oxidative stress increases TF transcription, stimulating backward motility.

Page 84, line 20:

Microcompetition between a GABP virus and TF increases the probability of being trapped in the subendothelial space. Denote the number of viral N-boxes with  $V_{Nbox}$ .  $[X]V_{Nbox}$  increases the inefficiencies in foam cell backward motility, denoted  $I$  in above clearance model.

Page 131, line 28:

Microcompetition decreases binding of GABP to the N-box.  $[X]$  Oxidative stress also decreases the binding of GABP to the N-box.  $[X]$  Therefore, microcompetition can be viewed as “excessive oxidative stress.” Some antioxidants reduce intracellular oxidative stress. These antioxidants stimulate the binding of GABP to the N-box attenuating the effect of microcompetition on transcription, resulting in slower progression of the microcompetition diseases.